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Biology of Asaccharolytic Black-Pigmented *Bacteroides* Species

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INTRODUCTION

Infections of mucous membranes are mixed infections (18), with members of the genus *Bacteroides* being the most commonly isolated anaerobes from these sites. In many of these instances, the suspected pathogens are species of the genus *Bacteroides* which produce brown to black colonies on blood agar media. These microorganisms are commonly referred to as black-pigmented *Bacteroides* (BPB). Members of this group are gram-negative, strictly anaerobic, nonmotile, nonsporeforming rods (53). Although BPBs have been isolated from a large number of clinical infections (12, 93, 94, 130, 170, 171, 217), mostly in association with other bacteria, very little is known about their significance or role in the infectious process. The pigmented *Bacteroides* now includes 10 species, 8 of which can be isolated from humans. Because most of these species were previously known as *Bacteroides melaninogenicus* (see references 50, 93, 142, 152, 156, 170), it has created a problem in the taxonomic interpretation of the literature prior to 1980, since *B. melaninogenicus* referred to several different species which are not included in the literature before 1980. Therefore, it is difficult to know which species were studied. At the present time, only three nonfermentative species of BPBs are described in the literature: *Bacteroides asaccharolyticus*, *Bacteroides endodontalis*, and *Bacteroides gingivalis*. (Note: Whenever possible, and appropriate, we will use the most accepted taxonomic designation for the members of the genus *Bacteroides*. When first discussed, we will use the original designation by the authors followed by our interpretation of that species, using presently accepted taxonomic criteria.)

In this review we have attempted to provide a useful synthesis of the literature relevant to the taxonomy, ultrastructure, physiology, serology, ecology, and pathogenicity of the asaccharolytic BPBs and to provide a perspective for their possible role in pathogenesis.

OVERVIEW OF THE TAXONOMY

In 1921, Oliver and Wherry (130) isolated a small anaerobic gram-negative rod from a variety of sites, including the oral cavity, urine, human feces, and respiratory tract, as well as from postsurgical infections. This rod, when grown on blood agar plates, produced colonies which were black in pigmentation. The pigment was considered to be melanin, and they named the culture *Bacterium melaninogenicum*. This bacterium was first described in the third edition of *Bergey's Manual of Determinative Bacteriology* (7) as *Haemophilus melaninogenicus* because better growth was obtained on solid medium containing X and V growth factors characteristic of the members of the genus *Haemophilus*.

Prévot in 1938 (140) concluded that the genus *Bacteroides*

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was invalid, and he regrouped several species into new genera and species. Prévot proposed that the name *Haemophilus melaninogenicus* be changed to *Ristella melaninogenica*. However, the name was not generally accepted, and the fifth edition of *Bergey's Manual* retained *Bacteroides melaninogenicus* (see Fig. 1). In 1947, Schwabacher et al. (153) proposed a new name for *B. melaninogenicus* based principally on pigmentation. Because the organism was classified by Wilson and Miles (224) in the group *Fusiformis*, Schwabacher et al. suggested *Fusiformis nigrescens*. However, *F. nigrescens* did not take precedence, and the seventh edition of *Bergey's Manual* (67) retained *Bacteroides melaninogenicus* as the proper description of the organism.

Although Gibbons and co-workers (16, 152) showed biochemical and immunological heterogeneity among strains of *B. melaninogenicus*, only one species of BPB was recognized until 1970: this was *B. melaninogenicus*. Along with an increased understanding of microbial physiology and the usefulness of end product analysis as a tool in taxonomy, it soon became clear that *B. melaninogenicus* could be taxonomically divided into several "subspecies" as a result of their fermentative activities (54). Therefore, the saccharolytic strains of *B. melaninogenicus* were divided into two subgroups: *B. melaninogenicus* subsp. *melaninogenicus*, which was strongly fermentative, and *B. melaninogenicus* subsp. *intermedius*, which was weakly fermentative. Asaccharolytic strains, that is, those which did not lower the pH of a glucose-based growth medium, were grouped in *B. melaninogenicus* subsp. *asaccharolyticus* (54). The studies of Finegold and Barnes (25) showed very clearly that the biochemical and genetic characteristics of the saccharolytic and asaccharolytic strains were sufficiently different so as to justify the elevation of the asaccharolytic subspecies to the species level. Further, Shah et al. (156) and van Steenbergen et al. (205) were able to separate the oral and nonoral *B. melaninogenicus* species based on their genetic heterogeneity, particularly among the asaccharolytic strains. This led Coykendall et al. (17) to propose the new species *B. gingivalis* for the asaccharolytic BPB strains isolated from oral sites. *B. asaccharolyticus* was retained for the nonfermentative *Bacteroides* sp. isolated from nonoral sites (Fig. 1).

Results from deoxyribonucleic acid hybridization experiments indicated little similarity between oral and nonoral types. The deoxyribonucleic acid base content of *B. gingivalis* varied from 46.5 to 48.4 mol% G+C, while that of *B. asaccharolyticus* varied between 49.2 and 53.6 mol% G+C (17). Table 1 lists the basic characteristics which distinguish the two species. Additional characteristics useful in distinguishing the two species include electrophoretic mobility of malate dehydrogenase (156), protein (176, 189) and cellular fatty acid (112, 155) profiles, and the composition of the mucopeptide (156, 224).

Recently, van Steenbergen et al. (210) reevaluated the two asaccharolytic BPB strains originally isolated by Sundqvist (G. Sundqvist, Ph.D. thesis, University of Umea, Umea, Sweden, 1976). These *Bacteroides* strains, which were originally referred to as *B. asaccharolyticus*, had little or no deoxyribonucleic acid homology with either authentic *B. asaccharolyticus* or *B. gingivalis*. van Steenbergen et al. (209) therefore proposed a new species for these strains: *Bacteroides endodontalis*. Although *B. endodontalis* resembles *B. asaccharolyticus* in several respects (Table 1), it can be distinguished from the nonoral strain and from other *Bacteroides* species by its lower G+C content and the absence of antigens common to other asaccharolytic BPBs, as well as by a different polyacrylamide gel electrophoresis protein profile (209). Importantly, all three asaccharolytic species can be rapidly identified by their sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles (209; A. C. R. Tanner, personal communication; D. Mayrand, unpublished results).

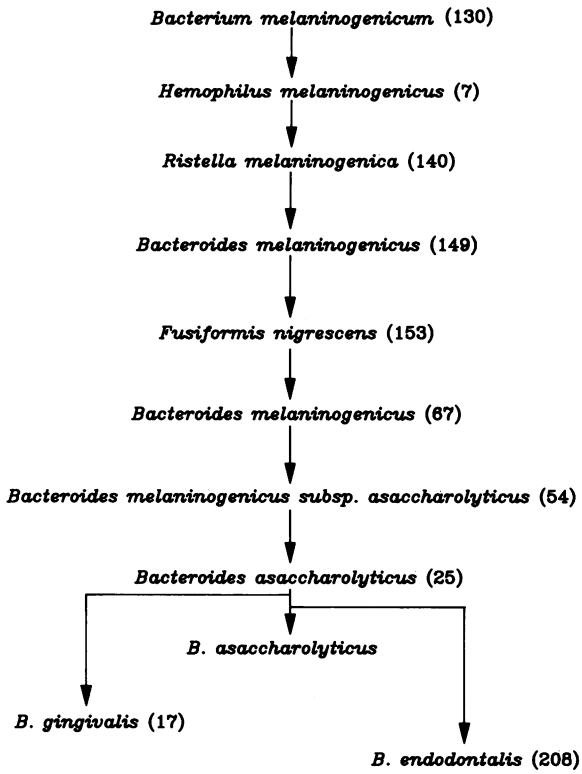

FIG. 1. Chronology for establishment of the asaccharolytic BPB species *B. gingivalis*, *B. asaccharolyticus*, and *B. endodontalis*. Numbers in parentheses are reference numbers. (Note that reference 208 should read 209.)

Asaccharolytic BPBs have also been isolated from other mammals. Syed (190) isolated several *Bacteroides* strains from dental plaque of beagle dogs. They were similar physiologically to the human strains; however, the dog strains were aerotolerant, catalase positive, and able to grow in media lacking hemin, menadione, blood, and reducing agents. Laliberté and Mayrand (75) found that all of the oral asaccharolytic BPB strains isolated from animals (dogs, cats, jaguar, and raccoon) were catalase positive, but otherwise similar to *B. gingivalis*. However, Kaczmarek and Coykendall (63) have reported that there are at least four genotypes of asaccharolytic BPBs in the mouths of dogs, and Love et al. (91) found five phenotypes from soft-tissue infections in cats.

ECOLOGY OF ASACCHAROLYTIC BPBs

With few exceptions (26, 76), very few members of the genus *Bacteroides* were actually isolated and described prior to 1970. The paucity of information regarding this genus was probably due to poor anaerobic culturing techniques as well as a poorly developed taxonomy. With the development of improved growth conditions, sampling, and identification methods, it became clear that these anaerobic organisms occupied a major fraction of the biomass of the human colon, as well as being associated with a large number of anaerobic infections. Studies related to the association of BPBs with

TABLE 1. Selected biochemical characteristics of the asaccharolytic BPB species *B. gingivalis*, *B. asaccharolyticus*, and *B. endodontalis*[a]

Characteristic	*B. asaccharolyticus*	*B. endodontalis*	*B. gingivalis*
Indole formation	+	+	+
Esculin hydrolysis	−	−	−
Starch hydrolysis	−	−	−
Metabolic products[b]	A, B, Ib, Iv, P	A, B, Ib, Iv, P	A, B, Ib, Iv, P, **Ph**
Catalase production	−	−	−
Hemagglutination	−	−	+
Trypsin activity	V[c]	−	+
Fucosidase	V	−	−
Chondroitin sulfatase	−	−	+
Gelatinase	+	+	+
Hyaluronidase	−	−	+
Oxygen tolerance (h)[d]	>24	6–24	>24
Vitamin K requirement	−	+	V
CO_2 requirement	−	+	V
Congo red inhibition[e]	+	−	−
Methylene blue[f]	−	+	−
G+C (mol%)	51–54	49–51	46–50
Cell size (μm)	0.8–1.5 by 1.0–3.0	0.4–0.6 by 1.0–2.0	0.5 by 1.0–2.0

[a] Characteristics useful in distinguishing the species are indicated in boldface type. Data are from references, 17, 63, 77, 81, 82, 101, 103, 104, 115, 141, 156, 165, 205, and 214.
[b] A, Acetic acid; P, propionic acid; Iv, isovaleric acid; B, butyric acid; Ib, isobutyric acid; Ph, phenylacetic acid.
[c] V, Variable results.
[d] Time required to destroy 90% of the initially viable cell population.
[e] Susceptibility to 50 μg/ml.
[f] Susceptibility to 100 μg/ml.

human diseases have recently been reviewed (164; A. J. van Winkelhoff et al., submitted for publication).

B. gingivalis

In the oral cavity, the asaccharolytic BPB *B. gingivalis* appears to be positively associated with several of the periodontal diseases (162, 164). For example, *B. gingivalis* is usually absent from healthy gingival sulci (161), while it may constitute <1 to 5% of the cultivable subgingival microbiota of individuals with gingivitis (164, 168, 219, 231). Its numbers increase significantly in adult periodontitis (21, 119, 173, 195, 219). As its species epithet implies, its common ecological niche is the gingiva, or periodontal pocket. However, it has occasionally been recovered from other sites in the mouth (31, 88, 231). In fact, *B. gingivalis* has recently been isolated from the tongue, tonsils, and saliva (van Winkelhoff et al., submitted). It has also been isolated occasionally from nonoral infections (135, 156); however, these latter observations may be open to some interpretation.

B. asaccharolyticus

B. asaccharolyticus is very widely distributed in both human and animal tissues and fluids. For example, it has been isolated from human feces (17, 20, 165) as well as from the cervix, ear tissue, umbilical cord, amniotic fluid, blood, empyema, peritoneal and pelvic abscesses, endometritis, and bite wound infections (17, 20, 35, 76, 165). Those strains isolated before 1980 and identified as oral *B. asaccharolyticus* are probably strains of *B. gingivalis*. To date, only two authentic strains of *B. asaccharolyticus* are known to be of oral origin, one being isolated from dental plaque (104) and the other from an oral submucous abscess (215).

B. endodontalis

B. endodontalis has only been isolated from mixed oral infections and predominantly from pyogenic infections of odontogenic origin (211) or dental root canal infections (42). While cultures of *B. endodontalis* have occasionally been isolated from dental plaque and oral mucosal surfaces (A. J. van Winkelhoff, personal communication), these sites do not appear to be its primary ecological niche. The limited number of *B. endodontalis* strains isolated makes it difficult to evaluate the clinical importance of this new species and its incidence in both oral and nonoral sites. Its isolation from oral infections suggests a possible association with the pathogenicity of such infections.

Experimental Infections Involving Asaccharolytic BPBs

Anaerobic bacteria comprise a large percentage of the oral and gut microbiotas, and because some of them possess several potentially pathogenic factors, it is not unreasonable to assume that they may be involved in the initiation of pathogenic processes. Often, infections at mucosal surfaces involve several bacterial species or genera that behave cooperatively or synergistically to produce sepsis. Because asaccharolytic BPBs are very often associated with mixed infections, a number of studies (24, 36, 94, 106, 109, 147, 170, 182, 191) have been initiated to determine whether, in fact, virulence of one species could be enhanced by combining it with other bacterial species in an animal model. The typical experimental mixed anaerobic infection with BPBs was first described by MacDonald et al. (94), and in most studies the experimental designs were very similar. Combinations of bacteria isolated from various sources (dental plaque, necrotic dental pulp, or periodontal pockets) have been tested for their capacity to induce abscess formation and transmissible infections when inoculated subcutaneously into guinea pigs. Results indicate that, except for one case (191), all of these experiments dealt with mixtures of anaerobes and facultative anaerobes. The studies showed a synergistic effect; e.g., individual bacterial species or isolates (including the BPBs) were not able to induce an infection, whereas mixtures of two or more bacteria did produce the effects.

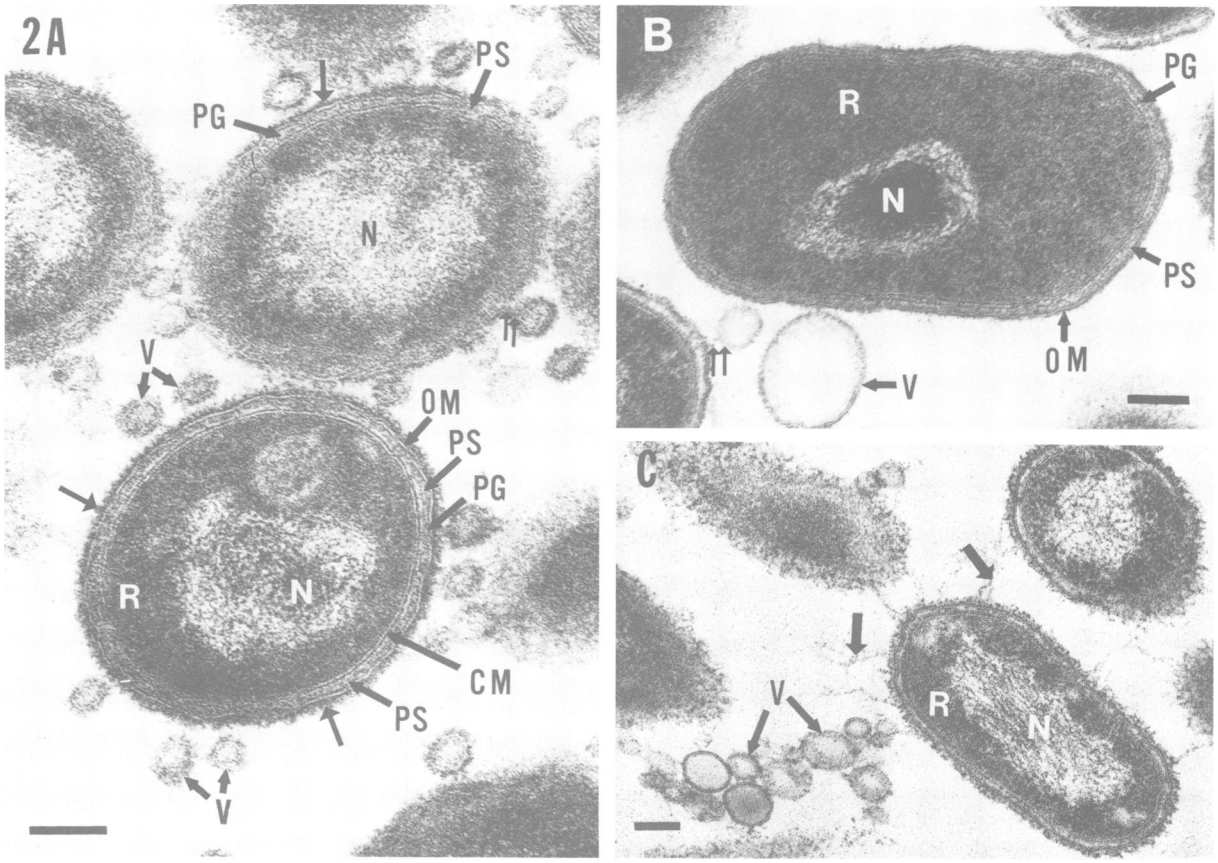

FIG. 2. Electron micrographs of (A) *B. gingivalis* ATCC 33277, (B) *B. asaccharolyticus* ATCC 25260, and (C) *B. endodontalis* H11a-e. The outer membrane (OM) is covered by an electron-dense capsule (arrows) and encloses a particulate periplasmic space (PS), both of which enclose the unit cytoplasmic membrane (CM) which encloses the electron-dense ribosomal region (R) and nucleoid (N). The thin peptidoglycan (PG) traverses the periplasm, while vesicles (V) are apparent in all species. The vesicles are clearly seen in panel A to be formed from the OM (double arrows). Note that the vesicles are also enclosed by a double membrane which encloses an electron-particulate core, probably of periplasmic origin. In panel C the capsule is seen to radiate from the surface of the OM (arrows). Bar, 100 nm.

Most of these experimental infections had another common trait: when BPBs were present in the "infectious mixture," it was possible to produce a transmissible infection. Importantly, when the BPBs, and particularly the asaccharolytic BPBs, were deleted from the infectious mixtures, a transmissible infection was not formed. The asaccharolytic BPBs, then, probably play a key role in mixed infections.

The nature of this synergistic infective mechanism is not fully understood. It has been shown, however, that naphthoquinone, a vitamin K-related compound, produced by associated bacteria can enhance the growth of *Bacteroides* species (93). Mayrand and McBride (106) found that succinate, produced by the fermentation of glucose by a facultative organism, can replace hemin as a growth factor for *B. gingivalis*. This relationship is not species specific since other bacteria can supply succinate to *B. gingivalis* (36, 106). More recently, another growth factor for *B. gingivalis* produced by *Wolinella recta* was identified as protoheme (38). On the other hand, once *B. gingivalis* is established, it is possible that other mechanisms such as hematin or bacteriocin production can help this species to suppress other bacterial species (192). In any case, it is clear that other factors such as interbacterial adherence and the cumulative toxic effects of metabolic products can take part in the initiation and development of the infectious process (36, 102, 166, 208). Other possible mechanisms of microbial synergy in polymicrobial infections have been extensively reviewed by Rotstein et al. (148).

ULTRASTRUCTURAL STUDIES

The first electron microscopic observations of BPBs were reported by Takazoe et al. in 1971 (194). In addition to a typical gram-negative morphology, they showed the presence of a capsule and fimbria-like structures in a pathogenic strain of *B. melaninogenicus* (*B. gingivalis*). Mansheim and Kasper (97) examined two strains (strains 376 and 382) of what is now known as *B. gingivalis* and one strain of *B. asaccharolyticus* (strain B536). Their electron microscopic observations of thin sections of the two species revealed them to be typical gram-negative bacteria: the cells contained an inner and outer cell membrane which was separated by a thin peptidoglycan layer (Fig. 2). Capsular material external to, and associated with, the outer membrane was also found in the oral strain of *B. melaninogenicus* subsp. *asaccharolyticus* (*B. gingivalis*) but not in the nonoral strain (*B. asaccharolyticus*). However, Mansheim et al. (99), using ruthenium red to stain acidic mucopolysaccharides, showed that *B. asaccharolyticus* B536 had some capsular material associated with its outer membrane. However, this "capsule" was not serologically cross-reactive with capsular antigens of the two *B. gingivalis* strains.

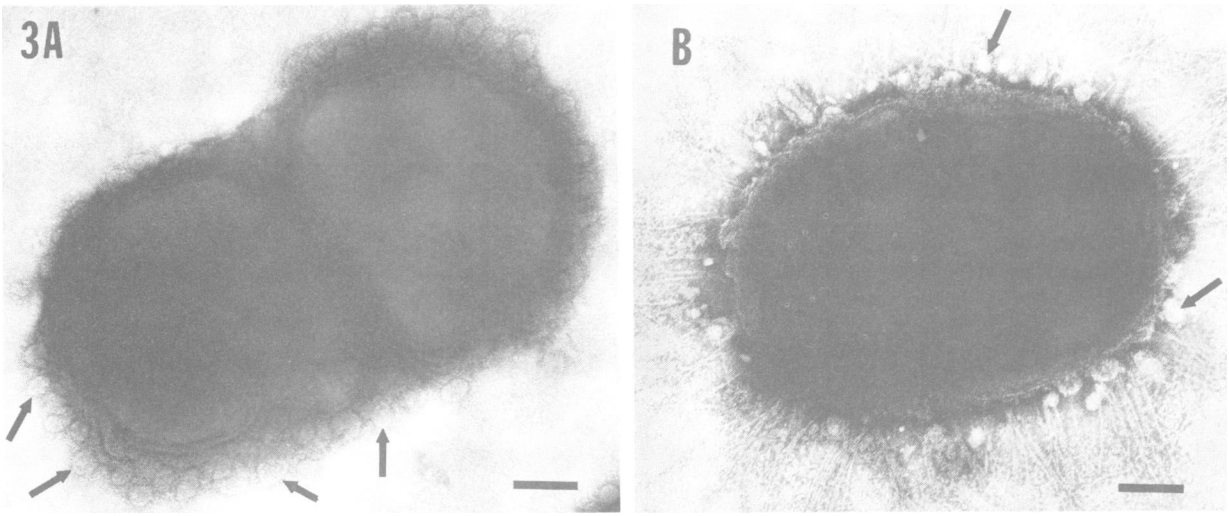

FIG. 3. Negative stained whole cells of (A) *B. gingivalis* W83 and (B) *B. gingivalis* CO. Numerous vesicles (arrows) line the surface of the outer membrane. Several vesicles appear to be in close contact with the outer membrane. Note in panel B the numerous fibrils (fimbriae) which emanate from the cell surface. Negative stain = 2% ammonium molybdate. Bar, 100 nm.

Detailed studies of the ultrastructure of BPBs were reported simultaneously by Listgarten and Lai (87) and Woo et al. (226). Both studies showed that these strains possessed a similar morphology but could vary with surface structures external to the outer membrane. Some *B. asaccharolyticus* strains exhibited a fine fibrillar ruthenium red staining matrix on their cell surface which appeared to form an interconnecting matrix between cells. *B. gingivalis* and *B. endodontalis* can also exhibit this matrix (Fig. 2A and C). Woo et al. (226), using critical point drying and scanning electron microscopy, showed that these fibrils emerged from the cell surface in intertwined strands (see Fig. 3B). More recently, Handley and Tipler (48) also demonstrated the presence of fimbriae and of an external layer outside of the outer membrane. The three asaccharolytic BPBs do not show crystalline external surface layers outside their outer membrane as exhibited by *W. recta* (74) and various nonpigmenting *Bacteroides* strains (41, 160).

In addition to a capsule, several *Bacteroides* species are capable of producing "vesicles" or "blebs" which are formed as a result of a pinching or budding of the outer membrane (39, 43, 87, 226) (Fig. 3 and 4). Growth studies have revealed that bleb formation may occur in response to environmental stress (71, 196). *Capnocytophaga* spp. (45, 138), *Actinobacillus actinomycetemcomitans* (73, 123), and *Cytophaga* spp. (90) have also been shown to form these vesicles. Their role in pathogenesis or virulence or both is under active investigation [see subsection, "Outer Membrane Vesicles (Blebs)"].

NUTRITION, PHYSIOLOGY, AND CELL COMPONENTS

The asaccharolytic BPBs are obligately anaerobic, and most of them require hemin and menadione (vitamin K) for growth (32). In most cases, a complex medium consisting of Trypticase (BBL Microbiology Systems), yeast extract, and mineral salts is used for growth (152). A survey of a variety of nonselective and selective media for *Bacteroides* spp. was compiled by Dowell and Lombard (18) and by Macy (95). The BPBs grow in the defined medium of Socransky et al. (169); however, the oral BPBs grow poorly in this highly complex mixture. Hunt et al. (57) have recently described a selective medium for the isolation of *B. gingivalis* which contains bacitracin, colistin, and nalidixic acid as selective agents. Results have indicated that this medium can support the growth of *B. gingivalis*, but inhibits the growth of the other two asaccharolytic BPB species. Of note, van Winkelhoff and de Graaff (212) and Sutter et al. (185) have recommended that vancomycin should not be used for the isolation of asaccharolytic BPBs, because most of the asaccharolytic BPBs are inhibited by 5 μg of this agent per ml.

The roles of hemin and vitamin K for the growth of asaccharolytic BPBs are not completely understood. Gibbons and MacDonald (32) have proposed that vitamin K functions as an electron carrier in electron transport. On the other hand, vitamin K has been found to stimulate synthesis of phosphosphingolipids in the cell envelope, suggesting a possible role in membrane permeability (84). Recent studies (215) have indicated that *B. asaccharolyticus* was capable of growth when either vitamin K or hemin was added to the growth medium. *B. gingivalis*, on the other hand, required hemin for growth (and some strains also require vitamin K), while *B. endodontalis* did not but required vitamin K. Succinate has also been shown to replace hemin or vitamin K as a growth factor for *B. melaninogenicus* (83) and *B. gingivalis* (36, 106).

The asaccharolytic BPBs all produce a dark brown-black pigment when grown for 6 to 10 days on blood agar plates. Early studies of the pigment by Oliver and Wherry (130) described it as melanin because of its apparent insolubility in many organic solvents. Further, the pigment was also considered to be extracellular. However, Schwabacher et al. (153) showed the pigment to be soluble in pyridine, and spectroscopic analysis revealed it to be the hematin derivative ferriprotoporphyrin. A series of studies continued to dispute the chemical structure of the pigment as well as its location (133), and it remained for Duerden (19) to demonstrate the intracellular, or at least cell-associated, nature of the pigment. He also determined the pigment to be water soluble, with spectrophotometric characteristics of a hemoglobin derivative. Shah et al. (154) unequivocally demonstrated that the hemoglobin derivative produced by *B. gingivalis* was protohemin with traces of protoporphyrin.

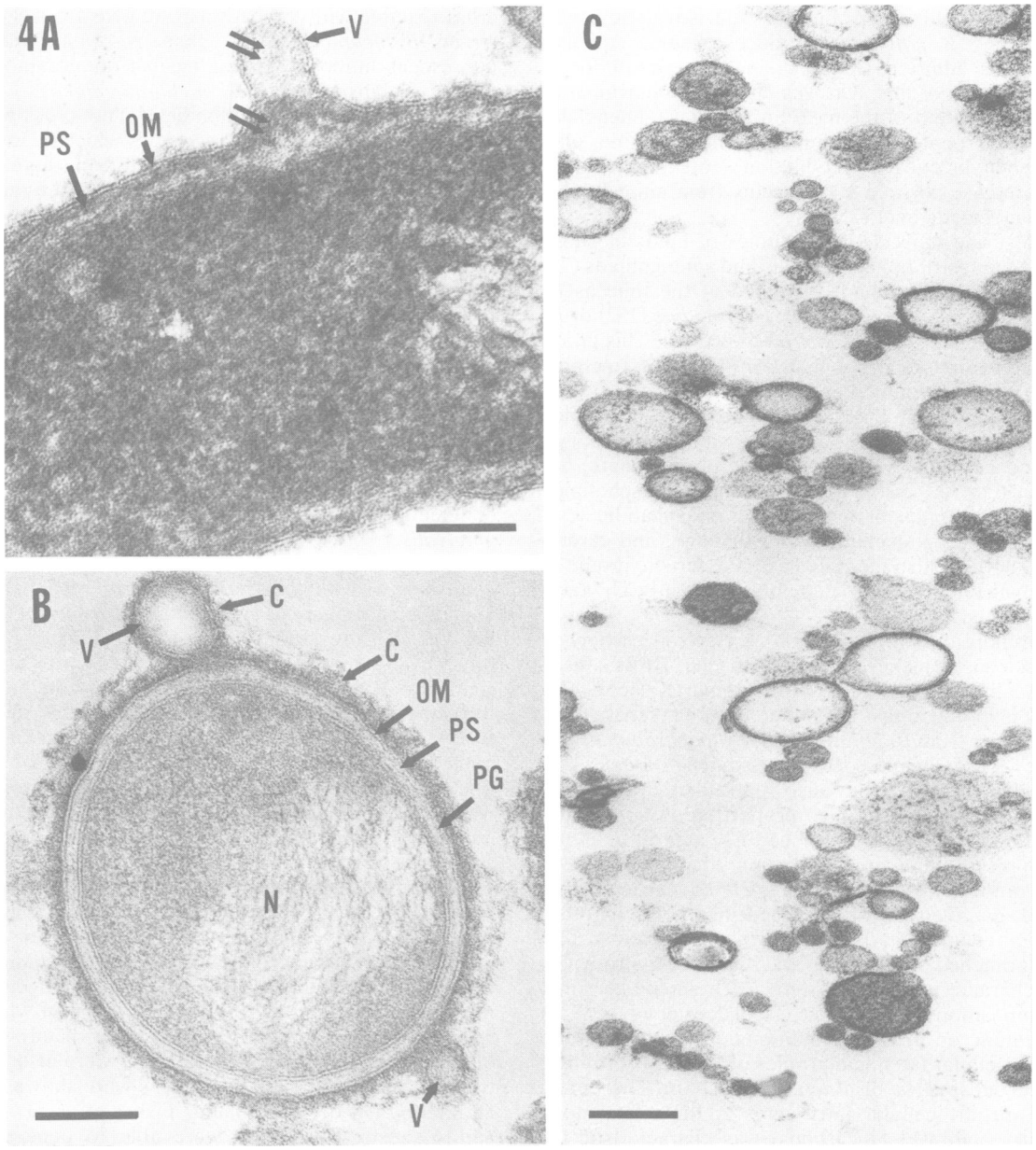

FIG. 4. Electron micrographs of thin sections of *B. gingivalis* W50 (A, B) and an isolated, enriched vesicle fraction of *B. gingivalis* W83 (C). The outer membrane (OM) encloses the electron-dense periplasmic space (PS). In panel A, a vesicle was in the process of formation (arrow). Note in panel A that what appears to be periplasmic material has been trapped in the developing vesicle (double arrow). In panel B, strain W50 was grown in low (0.4 μg/ml) hemin to enhance vesicle formation, and the capsule was stabilized with anti-whole-cell W50 antisera. A thick capsule (C) covers the outer membrane as well as the developing vesicles. In panel C, the enriched vesicle fraction consists of structures of various sizes (50 to 200 nm). In most instances the vesicles enclose an electron-dense material. Note in panel C that the vesicles appear to be devoid of a capsular covering. A, C, Lead citrate, uranyl acetate stained; (B) ruthenium red stained. Bar, 100 nm.

Gibbons and MacDonald (32) have suggested that the dark pigment is a mechanism of storage of hemin, a proposal consistent with the observations of Rizza et al. (142), who have shown that when *B. gingivalis* was grown in a medium containing a high concentration of hemin the cells were subsequently able to divide 8 to 10 times in a medium without hemin, suggesting the accumulation and storage of this compound.

While the asaccharolytic *Bacteroides* spp. may use hemin or protoheme in their electron transport system, it is not clear how adenosine 5′-triphosphate is generated via electron transport. Rizza et al. (142) have been able to demonstrate a membrane-bound respiratory system involving flavoprotein, cytochrome *c*, and a carbon monoxide-binding protein. Adenosine 5′-triphosphate in the asaccharolytic BPBs is probably obtained by the fermentation of peptides (216). When *B. gingivalis* W50 was grown under chemostat conditions (108), the cells were shown to preferentially utilize arginine, cystine, histidine, serine, and tryptophan as their sole carbon and energy source. It is interesting to note that glucose or other sugars inhibited the growth rate (85) of these asaccharolytic species.

The physiological end products of the three asaccharolytic BPBs are shown in Table 1. While the three species produce

acetic, propionic, butyric, isobutyric, and isovaleric acids, only one species, *B. gingivalis*, produces phenylacetic acid (63, 101, 209). In addition, Bourgeau and Mayrand (9) found that the production of this acid was directly proportional to the Trypticase content of the medium and that L-phenylalanine, as well as peptides containing L-phenylalanine, also stimulated phenylacetic acid production. Note that the asaccharolytic catalase-positive BPB strains from animals also produce phenylacetic acid (75).

The earliest and most detailed studies of lipids of BPBs involved the analysis of phospholipids and sphingolipids (72, 143, 220). The strain studied, identified at the time as *B. melaninogenicus*, may have been *B. intermedius* (152) or *B. asaccharolyticus* (see Table 6.5 of reference 53). This group of investigators first reported that 96% of the extractable fatty acids (chloroform-methanol extraction) were associated with the phospholipids, and phosphosphingolipids (ceramide phosphoryl ethanolamine, ceramide phosphorylglycerol, and ceramide phosphorylglycerol phosphate) accounted for 50 to 70% of the lipid phosphate. Phospholipids identified included phosphatidylglycerol, phosphatidic acid, phosphatidylserine, phosphatidylethanolamine, and cardiolipin. These authors also identified a characteristic profile of branched-chain, nonhydroxylated, predominantly C_{15} fatty acids.

The isoprenoid quinones function as electron carriers in several electron transport systems. In the BPBs, these compounds appear to be related to vitamin K (155). The number of isoprene units has been used to separate *B. asaccharolyticus* from *B. gingivalis*. *B. asaccharolyticus* has 9 isoprene units, while oral *B. asaccharolyticus* {*B. gingivalis*} has 10 units. They also found that *B. asaccharolyticus* strains exhibited a high proportion of isopentadecanoic acid and that the iso-branched C_{15} acid in *B. gingivalis* was present in a greater amount than the anteiso-branched compound. Lambe et al. (77) have shown that strains of *B. gingivalis* can be distinguished from the other BPBs by their cellular fatty acids. The fatty acid ratio of 14.3 comparing branched hydroxy C_{17} and branched hydroxy C_{15} acids was characteristic of *B. gingivalis*. It should be noted that the composition of cellular fatty acid does not depend on culture medium or growth conditions. The same group analyzed the cellular fatty acid profiles of 160 strains from 12 species and subspecies of *Bacteroides* (100). The actual amount of specific cellular fatty acids or the ratio among selected fatty acids (13:17 carbons) was characteristic for each species and permitted the development of an identification key for pigmented as well as nonpigmented *Bacteroides* species.

ANTIGENIC AND SEROLOGIC CHARACTERIZATION

With the development of an immunological technology for the identification of microorganisms, it became clear that immunology was a very powerful tool for taxonomic identification of procaryotes, as well as an excellent tool with which determine cell relatedness. Early serological studies (16) revealed that strains of *B. melaninogenicus* (including fermentative and asaccharolytic strains) were serologically heterogeneous and therefore represented a spectrum of serotypes. While many of the early studies found little or no serological relationships between BPB strains and other unrelated bacterial species (16, 99, 125, 218), Mansheim et al. (99) also found that a capsular antigen which they recovered from *B. asaccharolyticus* was not serologically cross-reactive with the capsular antigens from *B. gingivalis* strains {*B. melaninogenicus* subsp. *asaccharolyticus*}. Using fluorescent-antibody staining against one strain of each of the three subspecies of *B. melaninogenicus*, Lambe (76) demonstrated that these antibodies did not cross-react with a variety of other aerobes or anaerobes which he tested. Most of the *Bacteroides* strains isolated from human clinical specimens could be assigned or serogrouped by immunofluorescence and the serogroup could be correlated with the subsequent biochemical characterization of the three subspecies. A subsequent study by Lambe and Jerris (78) established that *B. intermedius* consisted of at least two serogroups, serogroups C and C-1. Individual conjugates as well as the polyvalent antibody-fluorescent conjugate could be used for the identification of these serogroups. The specificity of the monovalent antibodies was demonstrated by Mouton et al. (114), who showed that a commercial polyvalent conjugate consisting of three monovalent conjugates prepared against selected strains of *B. melaninogenicus*, *B. intermedius*, and *B. asaccharolyticus* was capable of detecting homologous strains but was not able to detect *B. gingivalis*.

The antigenic specificity of *B. asaccharolyticus* and *B. gingivalis* was demonstrated by Reed at al. (141), using immunoelectrophoresis and immunodiffusion. In their study, Reed et al. showed that none of the strains of *B. asaccharolyticus* (obtained from nonoral sites) was antigenically similar to *B. gingivalis*. In addition, no common antigens were observed between the asaccharolytic and saccharolytic BPB strains. Lambe et al. (77) and Poxton et al. (139), using other methods, confirmed and extended these observations. The antigenic specificity of the three asaccharolytic BPBs was recently reported by Okuda et al. (125), using both an immunodiffusion test and an indirect immunofluorescent-antibody assay.

B. gingivalis was determined by Parent et al. (136) to consist of at least two serogroups. By crossed immunoelectrophoresis, they analyzed the surface antigens of human and animal oral strains of asaccharolytic BPBs and identified both cross-reacting and serotype-specific antigens. The human biotype exhibited 25 surface antigens, of which 2 were specific for the biotype. On the other hand, the animal biotype had 12 surface antigens and 2 were also specific for the biotype. Four common antigens were shown to exist on all strains. On the other hand, Fisher et al. (J. Dent. Res. **65**:816, abstr. 817, 1986) were able to demonstrate two serogroups within the human strains of *B. gingivalis*, and these groups appeared to be correlated with virulence. A major protein band at 54 kilodaltons in the virulent strains was absent in the avirulent strains. This group also showed that immunofluorescent microscopic examination of pure cultures, using fully absorbed antisera, differentiated the *B. gingivalis* serogroups. Recently, Bramanti and Holt (J. Dent. Res. **66**:223, abstr. 930, 1987) indicated that there appeared to be a relationship between the pathogenicity of *B. gingivalis* strains and the electrophoretic patterns of cell envelope proteins. Virulent strains (W83 and W50) exhibited polypeptide bands at 56 and 49 kilodaltons, while the avirulent strain (strain 33277) showed a polyacrylamide gel electrophoresis pattern with bands at 72, 53, and 37 kilodaltons.

B. endodontalis does not share any common antigens with *B. gingivalis* or *B. asaccharolyticus* (209), and *B. endodontalis* is agglutinated by an antiserum raised against itself (215). van Winkelhoff et al. (213) have also recently demonstrated that strains of *B. endodontalis* could be divided into at least three serotypes (O_1K_1, O_1K_2, and O_1K^-), using

TABLE 2. Characteristics of infections produced by selected strains of *B. gingivalis*, *B. asaccharolyticus*, and *B. endodontalis* after subcutaneous injection[a]

Species/strain	Animal	No. of cells injected	Lesion[b]	No. of deaths/no. of animals tested
B. gingivalis				
W83	Mouse	7×10^9	Spreading	3/5
	Mouse	2×10^{10}	None	4/4
	Guinea pig	4.5×10^9	Spreading	3/3
W50	Mouse	7×10^9	Spreading	3/5
	Mouse	2×10^9	Spreading	1/4
	Mouse	1×10^9	Spreading	10/10
	Mouse	1×10^{10}	None[c]	0/3
381	Mouse	5×10^9	Localized	1/8
	Mouse	2×10^{10}	Localized	0/4
	Guinea pig	7×10^{10}	Localized	0/3
33277	Mouse	2×10^{10}	Localized	0/4
	Guinea pig	2×10^{10}	None	0/3
19A4	Guinea pig	1×10^{11}	None	0/3
HG184	Mouse	7×10^9	Localized	0/3
1112	Mouse	2×10^{10}	Localized	0/4
B. asaccharolyticus				
25260	Mouse	2×10^{10}	Flat lesion	0/4
	Mouse	5×10^9	Localized	0/3
27067	Mouse	5×10^9	Localized	0/3
B536	Mouse	5×10^9	Localized	0/3
B. endodontalis				
35406	Mouse	2×10^{10}	Flat lesion	0/4
H11a-e	Mouse	5×10^9	Flat lesion	0/4

[a] Data are from references 40, 108, and 206; Neiders et al., J. Dent. Res. **65**:208, abstr. 351, 1986; and van Steenbergen et al., submitted.
[b] Spreading, Spreading infection with exudate and and pus formation, necrosis of the skin, often fatal in 1 to 3 days; localized, localized abscess which can be 1 to 4 mm or more in diameter, necrotic or not, but not fatal; flat lesion, localized abscess without necrosis; none, no evidence of infection.
[c] Cells grown in the absence of hemin.

whole-cell agglutination. These results were based on the presence or absence of capsular material. When the capsule was removed, a common or O antigen was detected in all of the *B. endodontalis* strains.

PATHOGENICITY OF ASACCHAROLYTIC BPBs

With very few exceptions, members of the genus *Bacteroides* do not produce overt disease in animals and humans. While *B. fragilis* is capable of producing abscesses in a rat model (131) and of evading phagocytic mechanisms (157), most of the BPBs are not infectious for mice and guinea pigs even when injected in pure culture by various routes of infection (107, 206). Toxins other than the lipopolysaccharide (LPS) have not been isolated. In fact, only a limited number of BPB strains can produce infections which can be transmitted to a second animal (66, 93, 182, 194). Mayrand and McBride (106) have shown that a nonvirulent *B. gingivalis* strain is infective only when injected into a guinea pig along with a hemin-agar mixture. The agar appears to retard the rapid diffusion of the hemin out of the system. McKee et al. (108), however, were able to show that, when the pathogenic strain of *B. gingivalis* W50 was grown in a hemin-deficient medium, it produced progeny which were avirulent when injected subcutaneously into mice. In a comparison of the virulence of several BPBs in a mouse model, van Steenbergen et al. (206) showed that *B. gingivalis* strains were generally more virulent, causing a severe phlegmonous abscess, while *B. assacharolyticus* strains produced only localized abscesses. These results were confirmed and extended by Neiders et al. (J. Dent. Res. **65**:208, abstr. 351, 1986), who also determined the relative virulence of *B. gingivalis* in a BALB/c mouse model. They investigated the difference between invasive and noninvasive *B. gingivalis* strains as a function of their proteolytic activity. Azocoll degradation was followed under reduced conditions, and it was shown that Azocoll was more readily degraded by invasive *B. gingivalis* strains than by noninvasive ones. There were, however, no differences in proteolytic activity against other substrates, such as Azocasein, L-arginine-β-naphthylamide hydrochloride, *N*-carbobenzoxy-glycyl-glycyl-L-arginine-β-naphthylamide hydrochloride, and the substrates found in the API-ZYM (Analytab Products) system.

Grenier and Mayrand (40), using a guinea pig model, also found two subgroups within strains of *B. gingivalis*. Several of the *B. gingivalis* strains were virulent in pure culture, and all but one of these strains were more collagenolytic than those which failed to cause lesions. Table 2 shows the virulence characteristics of virulent and avirulent *B. gingivalis* strains. Fisher et al. (J. Dent. Res. **65**:816) also recently described two serogroups (serogroups A and B) of *B. gingivalis*. *B. gingivalis* serogroup A were isolated from periodontally healthy subjects and included strains which were less virulent in a mouse lethality test than strains of serogroup B, which were isolated from subjects with periodontitis. How-

ever, it is not clear yet whether *B. gingivalis* strains recovered from periodontal disease "active sites" are always infectious or even invasive in animal models. Further, there are little data which correlate virulence with the presence of specific biochemical properties, such as proteolytic-collagenolytic activity.

FACTORS AFFECTING VIRULENCE

Since asaccharolytic BPB strains are very often associated with human opportunistic infections, and the pathogenic potential of some of them has been clearly demonstrated in experimental infections, factors which may contribute to the virulence of these bacteria have recently been under intensive investigation (55, 193).

Adherence

Within a dynamic system, the ability to attach to mucosal surfaces is, in many instances, a prerequisite for both colonization and disease initiation (6, 34). Bacterial cell surfaces have associated with them specific adhesins which are responsible for attachment to specific host receptors (6, 23, 55). Gram-positive cells, for example, streptococci, attach to host cells and other microorganisms by specific fimbriae and lipoteichoic acid (6). Gram-negative bacteria, on the other hand, may have several adhesins on their surface which are responsible for attachment to specific host receptors (22, 56, 124). These adhesins include type-specific pili or fimbriae, hemagglutinins, and other surface-binding proteins.

Fimbriae. Most, but not all, of the BPBs have pili or fimbriae on their outer membrane (10, 48, 126, 167, 227). The fimbriae from *B. gingivalis* have been isolated and purified, and their morphological, immunological, and chemical properties have been characterized (229, 230). They are heat-stable, thin, curly filaments, approximately 5 nm in width. The fimbriae subunit (fimbrilin) has an apparent molecular migration of 43,000 and a primary structure which is different from that of other gram-negative bacteria (230). Yoshimura et al. (230) have also shown that native fimbriae and denatured fimbrilin differ greatly in their immunological properties in that they show little cross-reactivity. The fimbriae of asaccharolytic *Bacteroides* spp. were originally thought to confer hemagglutinating activity (128). However, pure preparations of the fimbriae of *B. gingivalis* did not show either hemagglutination activity or hemagglutination inhibitory activity (229).

Functionally, Slots and Gibbons (167) reported that *B. gingivalis* had the capacity to attach to both buccal and crevicular epithelial cells as well as to the surface of gram-positive bacteria. They also showed that, while saliva and serum had adherence-inhibiting effects on the attachment of *B. gingivalis* to erythrocytes and epithelial cells, these fluids did not affect its attachment to other bacteria. This suggests that there may be two kinds of fimbriae on the surface of *B. gingivalis*, an observation supported by the fact that both hemagglutinating strains of *B. gingivalis* and strains lacking hemagglutinating activity (fermentative strains of BPBs such as *B. intermedius* and *B. melaninogenicus*) exhibit fimbriae on their surface. In addition, the hemagglutinating activity of partially purified fimbrial preparations was destroyed by heating at 60°C for 15 min (167), whereas the fimbrial preparation of Yoshimura et al. (229) was heat resistant (incomplete dissociation at 80°C for 20 min in sodium dodecyl sulfate).

Recent studies (Suzuki et al., manuscript in preparation) with agglutination and immunodiffusion assays have shown that approximately 50% of the clinical isolates of *B. gingivalis* examined possessed fimbriae; however, all of these strains hemagglutinated erythrocytes equally well. It is possible that these techniques were such that they could not "see" fimbriae on all of the *B. gingivalis* strains, or that some strains were examined at a different growth phase in which fimbriae were absent, or that the antifimbrial antiserum was too specific and not able to identify all of the *B. gingivalis* fimbriae on these strains. It has also been shown that sera from patients with periodontal diseases reacted strongly with fimbriae by Western blotting (immunoblotting) analysis (Yoshimura et al., submitted for publication).

Hemagglutinating activity. *B. gingivalis* can hemagglutinate erythrocytes isolated from various animals (10, 107, 125, 128, 165, 167, 192). In fact, this property is an important taxonomic character which can distinguish *B. gingivalis* from *B. endodontalis* and *B. asaccharolyticus* (104, 107, 125, 165, 209). However, confusion still exists as to the type of receptor responsible for this activity. Extraction of surface components of *B. gingivalis*, such as capsular polysaccharide or LPS, did not exhibit hemagglutinating activity and did not inhibit hemagglutination (126). On the other hand, results by Boyd and McBride (10) indicated that the hemagglutinating activity was associated with low-molecular-weight LPS, protein, and loosely bound lipid. They also showed that removing fimbriae from *B. gingivalis* had no effect on the hemagglutinating activity of whole cells. More recently, Inoshita et al. (61) isolated an exohemagglutinin from the culture medium of *B. gingivalis*. It consisted of several proteins but no detectable LPS. Okuda et al. (129) also purified a hemagglutinin from *B. gingivalis* which consisted of at least two protein components and some loosely bound lipid components. It is interesting that the inhibition of attachment of *B. gingivalis* to erythrocytes or other bacterial cells is mediated by a low concentration of L-arginine (39, 61, 129). Results obtained by Inoshita et al. (61) suggest that the inhibitory effect of arginine on hemagglutination can be attributed to the guanido group of arginine. It is possible that arginine functions as a contact residue between the bacterial cell receptor and its counterpart on the erythrocyte during agglutination.

Other possible binding adhesins. Slots and Gibbons (167) and Okuda et al. (126) have shown that *B. gingivalis* could also attach to human epithelial cells, as well as to gram-positive bacterial species (10, 167). Boyd and McBride (10) indicated that a bacterial aggregating component isolated from the outer membrane of *B. gingivalis* was composed of protein, carbohydrate, and a high-molecular-weight LPS fraction.

Lantz and co-workers (79) found that *B. gingivalis* also binds to fibrinogen. Binding to this substrate was found to be rapid, highly specific, and saturable. This group also showed that *B. gingivalis* possessed a cell-associated fibrinogen-degrading thiol protease. These authors, as well as McKee et al. (108), have pointed out the importance of assaying binding and degradation activities of *B. gingivalis*. Depending on environmental conditions, the relative importance of these activities could function to assist the cells either to colonize a surface by providing the bacterial cells with key nutrients or by protecting the bacteria from host defense mechanisms.

The *B. asaccharolyticus* strains so far examined do not

appear to adhere to either human epithelial cells (126, 167) or the surfaces of gram-positive bacteria (167).

Capsules of Asaccharolytic BPBs

Bacterial capsules have various functions: they can serve as physicochemical barriers for the cell, they provide protection against desiccation by binding water molecules, and they are antiphagocytic in that they function to avoid engulfment by polymorphonuclear leukocytes (PMNLs). Capsules function to prevent hydrolytic degradation of microorganisms if engulfed by PMNLs. Capsules may also promote attachment of bacteria to other bacteria. The role of encapsulated anaerobic bacteria in synergistic infections has been reviewed recently by Brook (11).

Electron-dense material, approximately 15 nm thick, has been observed to cover the outer membrane of a number of *B. gingivalis* strains (48, 87, 96, 226; Fig. 4B). Woo and Holt (unpublished data) have removed this material from *B. gingivalis* 381 with hot formamide and determined it to consist of a polysaccharide heteropolymer. Okuda and Takazoe (127) have shown that an encapsulated, virulent, but nonhemagglutinating strain of *B. melaninogenicus* was more resistant to phagocytosis and killing by PMNLs than was a similar but noncapsulated strain. Furthermore, phagocytosis and phagocytic killing of *Staphylococcus aureus* were inhibited when extracted capsular material from the *B. melaninogenicus* strain was added to the system. van Steenbergen et al. (207) have confirmed and extended these observations by using the technique of chemiluminescence, in which a biological signal is produced by polymorphonuclear granulocytes after they have phagocytosed bacteria. They showed that the virulent strains of *B. gingivalis*, W83 and 50, were more resistant to killing by human serum and by PMNLs plus serum, and they showed lower chemiluminescence values than those strains which were less virulent, such as strains 376 and HG185 (van Steenbergen et al., submitted for publication). The virulent strains did not autoagglutinate, had a thicker capsule, and were much more hydrophilic than the less virulent strains. Their results indicate that the observed difference in virulence is due in part to a difference in capsular structure.

It seems that capsular material from the BPBs can inhibit their own phagocytic killing as well as that of facultative anaerobes (58, 59) and aerobic bacteria (118). Capsular material could also function to resist serum bactericidal systems involving complement. Sundqvist and Johansson (183) showed serum resistance for *B. gingivalis* strains, whereas other BPBs were killed by serum.

There are very little data concerning the capsules of *B. asaccharolyticus* and *B. endodontalis*. *B. asaccharolyticus* possesses an extracellular electron-dense capsule; however, it was more fibrous and loosely bound to the outer membrane than that observed in *B. gingivalis* (see Fig. 2e and 3b of reference 226 and Fig. 1 of reference 96). Some strains of *B. endodontalis* have also been reported to possess an electron-dense layer associated with the outer membrane. While *B. endodontalis* is also capable of resisting phagocytosis (178), the role of the capsule as an antiphagocytic structure in this species still remains to be determined. The capsule of *B. endodontalis* has also been shown to provide some serospecificity. van Winkelhoff et al. (213), for example, have been able to distinguish three serogroups within this species according to capsular antigens.

That *B. endodontalis* HG182 (Sundqvist strain BN11a–f) is more resistant to dyes and other inhibitory agents than other strains of the same species (104) and that it can induce transmissible infections when it is part of a mixed culture (182) can be taken as evidence that the particular capsule exhibited by this strain plays a role in infections.

LPS

The LPS of gram-negative bacteria is composed of three covalently linked parts: the lipid A moiety embedded in the outer membrane, a core polysaccharide found at the outer membrane surface, and the polysaccharide O antigen which extends from the outer membrane into the surrounding environment.

The LPS of the outer membrane complex of BPB does not have the same characteristics as that of other gram-negative bacteria (51, 97, 98). These "atypical" LPS lack heptose and 2-keto-3-deoxyoctonate (52, 98, 116). Fatty acids found in greatest abundance were hexadecanoic acid (77, 98) and an iso-branched C_{17} hydroxy fatty acid (77). No evidence for β-OH-myristic acid was observed (98). Rhamnose, mannose, galactose, and glucose were the neutral sugars detected, and glucosamine was the predominant amino sugar (98, 116).

Variations in the chemical composition, as well as the biological activity, of the LPS as measured by chicken embryo lethality exist among strains of *B. gingivalis* and between *B. gingivalis* and *B. asaccharolyticus* (98). By classical endotoxin assays (i.e., *Limulus* lysate assay or Schwartzman test), the LPS from the BPBs show very little endotoxic activity (51, 97, 186, 187).

Apart from its low endotoxic activities, the LPS from *B. gingivalis* has been shown to possess significant mitogenic activity (70). Bom-van Noorloos and co-workers (8) have shown that whole cells of *B. gingivalis* or its purified LPS stimulated bone resorption (60, 111, 116, 188). Millar et al. (111) also showed that the *B. gingivalis* LPS was capable of inhibiting bone collagen formation. Therefore, while several of the BPB LPS may be weak endotoxins, or may even be inactive or only weakly active in bone resorption, they may actively interfere with collagen formation. This role of LPS as an inhibitor of bone synthesis has not been widely studied.

In addition to its ability to function as both an inducer of bone resorption and an inhibitor of bone (collagen) formation, the *B. gingivalis* LPS has been shown to function as an inducer of interleukin-1 production. Through inflammatory reactions, interleukin-1 has been postulated to play a role in the pathogenesis of adult periodontitis (46). LPS from *B. gingivalis* has been shown to inhibit gingival fibroblast proliferation (80), an observation which could be significant to the destruction of connective tissue.

Outer Membrane Vesicles (Blebs)

Several gram-negative bacteria have been shown to form membranous extensions or outgrowths of the outer membrane during in vitro growth (65, 73; Fig. 3A and B). These vesicles or blebs appear to be similar, if not identical, to the outer membrane (Fig. 4A). A large body of information already exists relevant to these structures in the oral bacteria (39, 45, 73, 87, 108, 123, 138, 156). The vesicles are morphologically similar to the ones found in dental plaque (44). It is not clear if *B. asaccharolyticus* strains form these vesicles, but strains of *B. endodontalis* have similar structures (43).

The production of vesicles by *B. gingivalis* has been reported by several groups (39, 108, 136, 223). McKee et al. (108) showed that *B. gingivalis* W50 grown under hemin limitation produced large numbers of vesicles and possessed few fimbriae per cell. The number of fimbriae may be correlated with infectivity, since those cells which contained

small numbers of fimbriae were less infective in a mouse model, while cells grown in excess hemin were heavily fimbriated and had fewer vesicles, but caused 100% mortality in mice. Although it is not known whether the vesicles are shed into the periodontal pocket, they have been found in the pus of guinea pigs infected with a mixture of oral bacteria and *B. gingivalis* (36). The vesicles have been shown to exhibit proteolytic and collagenolytic activities, as well as to be capable of hemagglutinating erythrocytes (39). The vesicles also promote bacterial adherence between homologous *B. gingivalis* strains in addition to mediating attachment between non-coaggregating bacterial species (39).

While the role of vesicles in pathogenesis is unclear, there is a growing body of literature which indicates that they function as a virulence factor. In addition to their small size (50 to 150 μm in diameter; Fig. 4C), they are produced in large numbers. Their size could easily permit them to cross epithelial barriers that are otherwise impermeable to whole cells. These structures may have been responsible for the immunofluorescence seen by Pekovik and Fillery (137) at the surface as well as in the interstitial space between epithelial cells. The vesicles could serve as a vehicle for toxins and various proteolytic enzymes, as well as indirectly extending the bacterial cell's capacity to obtain nutrients. These membranous vesicles could also compete for antibodies and thus impede the specific antibacterial immune defense.

Enzymatic Activities Associated with Tissue Destruction

Roeterink et al. (145, 146) observed the effects of various BPB strains on the palate of the rat and on the hind foot of the mouse. Histologically, it appears that *B. gingivalis* causes its greatest damage to both soft and hard tissues. The exact mechanism by which the BPBs destroy tissue is still controversial (27, 86, 151); however, these bacteria or their products or both must in some way penetrate or invade the gingival connective tissues to initiate tissue destruction. Collagen (mostly of type 1) is the major constituent of the gingival connective tissues (28), and while this triple-helix protein is resistant to a wide variety of proteolytic enzymes, it is degraded by bacterial and tissue collagenases, both of which occur in the mouth (30, 89).

Some types of periodontal diseases are characterized by the loss of gingival connective tissues (134). The capacity of *B. gingivalis* to degrade collagen was first shown by Gibbons and MacDonald (33), who found that the collagenase enzyme was not affected by high salt concentrations and functioned optimally under neutral conditions. The enzyme was found to be synthesized intracellularly, with all of the enzyme activity being cell associated. Later, Hausmann and Kaufman (50) showed that the collagenase of *B. melaninogenicus* was tightly bound to a particulate fraction of a culture autolysate. Lantz and her associates (J. Dent. Res. **62**:289, abstr. 1076, 1983) have some evidence which indicates that the enzyme may be contained within the periplasmic space. Maximum enzymatic activity occurred when the cells were grown in a peptide-deficient medium or when cultures reached stationary growth phase (107, 144). While some collagenase activity was found in the culture supernatant, it was probably a result of cell autolysis (33, 107, 144). Collagenolytic activity was found to be stimulated by reducing agents such as dithiothreitol and cysteine (49) and was inhibited by antipain, chymostatin, thiol-protease (197), serum (144), ethylenediaminetetraacetate (105, 144), and hydrogen peroxide (50, 179).

As opposed to eucaryotic collagenases which cleave undenatured collagen at a single site, the collagenase from *B. gingivalis* hydrolyzes collagen into small peptides (144, 197; D. Grenier, Ph.D. thesis, Laval University, Quebec City, Quebec, Canada, 1986). Contradictory results for collagenolytic activity of asaccharolytic BPBs may be due to the different experimental designs used. Mayrand et al. (107), for example, showed that the saccharolytic BPBs lacked collagenase activity when the cells were grown in a supplemented Trypticase-yeast extract medium. Other workers (144, 175), using different growth media, techniques, and strains, found clinical specimens as well as several nonpigmented *Bacteroides* spp. to be collagenolytic. More recently, Mayrand and Grenier (105), van Steenbergen and de Graaff (203), and Sundqvist et al. (179) showed that *B. gingivalis* was the only BPB species which had collagenase activity. Mayrand and Grenier (105) also indicated that the degradation of collagen by the other *Bacteroides* species examined was the result of the action of nonspecific proteases, since the collagenolytic activity of *B. asaccharolyticus* was (i) not inhibited by ethylenediaminetetraacetate at 25 or 37°C, (ii) completely inhibited by 5 mM phenylmethylsulfonyl fluoride at 25 and 37°C, (iii) very low (<10%) at 25°C, and (iv) comparable to the activity found for a commercial protease control under the same conditions. Therefore, while *B. asaccharolyticus* and *B. endodontalis* can cause limited collagen degradation (105, 203), they do so by the action of nonspecific proteases rather than a collagenase (105, 179).

Enzymatic Activities That Can Perturb Host Defense Mechanisms

Some clinically important bacteria produce proteolytic enzymes that are capable of degrading most serum proteins involved in host defense against microbial infections. For example, both secreted and cell-bound immunoglobulin proteases, when active within a tissue (i.e., periodontal tissue), can interfere with the protective action of antibodies. The proteolytic destruction of immunoglobulins can result in an increase in bacterial adherence, a decrease in bacterial lysis due to complement, and a reduction of phagocytosis, as well as a decrease in antibody-neutralizing ability against toxins and enzymes. Degradation of immunoglobulins can thus paralyze locally the host defense mechanisms and may permit bacterial invasion of tissues. Several BPB strains can degrade immunoglobulins in vitro (68, 181). A vast majority of these BPBs and the three asaccharolytic *Bacteroides* species in particular can completely degrade the immunoglobulin molecules (68) and use the resulting fragments for their own growth (Grenier, Ph.D. thesis). However, no evidence of immunoglobulin degradation in tissues has been established.

Strains of *B. gingivalis* have been shown to resist phagocytosis even in the presence of specific antibodies and factor C3 of the complement system (178). Recently, Sundqvist et al. (180) showed that *B. gingivalis* W83 was able to degrade complement proteins C3 and C5 from guinea pig serum both in vitro and in vivo. By degrading complement and immunoglobulins, *B. gingivalis* may evade the phagocytic host defense.

B. gingivalis possesses specific proteases capable of degrading human plasma proteinase inhibitors (13, 120). These proteins probably contribute to host defense by neutralizing bacterial proteolytic enzyme activity. However, the major function of these proteinase inhibitors may be to modulate the activity of proteinases released by PMNLs (174). The proteolytic activity of *B. gingivalis* may thus permit a greater tissue destruction and favor a rapid progression of disease.

TABLE 3. Enzymatic activities of *B. gingivalis*, *B. asaccharolyticus*, and *B. endodontalis*[a]

Enzyme activity or substrate	*B. asaccharolyticus*	*B. endodontalis*	*B. gingivalis*
Gelatinase	+	+	+
Collagenase	V	−	+
Azocoll	−	−	+
Fibrinogen	ND	ND	+
N-Carbobenzoxy-Gly-Gly-Arg	−	ND	+
Aminopeptidase	+	ND	+
Trypsinlike	−	−	+
Fibronectin	+	ND	+
Phospholipase A	ND	ND	+
Acid phosphatase	+	+	+
Alkaline phosphatase	+	ND	+
Chondroitin sulfatase	V	ND	+
Hyaluronidase	V	−	+
Heparinase	−	−	+
Fibrinolysin	V	−	+
Keratinase	−	−	+
Deoxyribonuclease	+	+	+
β-Lactamase	−	−	−
Elastase	−	−	−

[a] +, Activity detected; −, no activity detected; V, variable results; ND, not done. Data are from references 3, 79, 81, 104, 105, 107, 110, 121, 144, 163, 175, 177, 203, 215, 221, 222, and 228.

B. asaccharolyticus and *B. endodontalis* do not seem to possess such ability, although only one strain of each species has been assayed (13).

B. gingivalis also possesses significant fibrinolytic activities (79, 121, 150, 222; P. A. Mashimo and J. Slots, J. Dent. Res. **62**:663, abstr. 123, 1983), providing it with the potential to invade tissues. *B. gingivalis* has been found to be very effective at degrading the iron transport plasma proteins such as albumin, haptoglobin, hemopoxin, and transferrin (14). Therefore, the highly proteolytic strains of *B. gingivalis* have the potential to degrade a large number of proteinaceous substrates to smaller peptides which may then be transported into the cell and used to satisfy its metabolic requirements. Several proteases from *B. gingivalis* have already been purified and characterized (1, 29, 132, 201, 228). Table 3 lists the known proteolytic activities of the three asaccharolytic BPB species, and Table 4 shows the enzymatic activities measured by the API ZYM system. However, it is important to note that most of these activities have only been shown in vitro, and it has yet to be demonstrated whether any of these occur in vivo.

B. gingivalis also elaborates superoxide dismutase and possibly a nicotinamide adenine dinucleotide, reduced form, peroxidase which may help the organism to resist the deleterious effects of oxygen and hydrogen peroxide (2). This may be an important virulence factor as this bacterium is able to colonize periodontal pockets and has the potential to invade gingival tissues.

Toxic Products

Strains of the three asaccharolytic BPBs, or culture filtrates of these bacteria, are cytotoxic for the African green monkey kidney (Vero) cell line (37, 65, 198, 203; H. Birkedal-Hansen et al., J. Dent. Res. **61**:192, abstr. 125, 1982). Further, butyrate and propionate, the characteristic end products of metabolism of the asaccharolytic BPBs, are potent inhibitors of various cultured human or animal cell lines (35, 159, 200, 204). Van Kampen et al. (202) have recently shown that proteoglycan production by chicken embryonic chondrocytes is inhibited when these cells are in contact with a culture filtrate of *B. gingivalis*. In this case, the toxic effects due to fatty acids have been excluded so that other, yet unknown factors may be implicated in the inhibition of proteoglycan synthesis. Other potentially toxic factors include the production of indole and ammonia (92) and volatile sulfur compounds including hydrogen sulfide, dimethyl disulfide, and methylmercaptan (198). *B. asaccharolyticus* and *B. endodontalis* produce the same sulfur compounds and may have the same toxic potential. Finally, it is important to note that *B. gingivalis* releases a substance which induces collagen breakdown in cultures of rat mucosal keratinocytes seeded on a lawn of reconstituted collagen fibrils (H. Birkedal-Hansen, J. Dent. Res. **62**:101, abstr. S51, 1987). This effect was shown to be the result of two distincts events: induction of secretion of procollagenase and the activation of this enzyme. The two events were triggered by two distinct compounds, both of which had proteolytic activity. These results suggest that *B. gingivalis* has the potential to destory periodontal connective tissues directly (with its own collagenase) or indirectly by inducing the secretion of host collagenase in the absence of local immune responses.

Despite the fact that asaccharolytic BPBs present a large array of putative mechanisms which may induce breakdown of tissues, it has been postulated that several prerequisites must be fulfilled to initiate a destructive phase (172). The multiplicity of these prerequisites makes it clear that the proper combination of factors needed for tissue destruction must occur rarely. In this context it is also clear that adherence and activities that help the bacteria to survive and multiply (enzymatic activities to obtain small peptides, for example) should be considered as the most important.

TABLE 4. Enzymatic activities of *B. gingivalis*, *B. asaccharolyticus*, and *B. endodontalis* as measured by the API ZYM system[a]

Enzyme activity	*B. asaccharolyticus* (n = 13)	*B. endodontalis* (n = 15)	*B. gingivalis* (n = 44)
Alkaline phosphatase	$+^{w}$	+	+
Butyrate esterase	V	V	V
Caprylate esterase	$+^{w}$	$+^{w}$	$-^{w}$
Myristate lipase	−	−	−
Leucine aminopeptidase	V	−	−
Valine aminopeptidase	−	−	−
Cysteine aminopeptidase	−	−	−
Trypsin	−	−	+
Chymotrypsin	−	−	$-^{w}$
Acid phosphatase	$+^{w}$	+	$+^{w}$
Phosphoamidase	$+^{w}$	$+^{w}$	$+^{w}$
α-Galactosidase	−	−	−
β-Galactosidase	−	−	−
β-Glucuronidase	−	−	−
α-Glucosidase	−	−	−
β-Glucosidase	−	−	−
β-Glucosaminidase	−	−	V
α-Mannosidase	−	−	−
α-Fucosidase	V	−	−

[a] +, Strong activity in all strains; −, all strains negative; $+^{w}$, all strains positive, some with a weak activity; $-^{w}$, most strains negative, some with a weak activity; V, variable results. Data are from references 81 and 163 and van Winkelhoff (Ph.D thesis).

ANTIMICROBIAL SUSCEPTIBILITY

Antibiotic susceptibility data on the three asaccharolytic BPB species is still equivocal. This is because essentially all of the information on antibiotic susceptibility comes from only a few isolates and in most cases is the result of studies with different assay techniques. It is therefore impossible to compare these data in a meaningful way. Because the BPB species are often part of mixed infections, and *B. gingivalis* and *B. endodontalis* have been associated with oral diseases (A. J. van Winkelhoff, Ph.D. thesis, Free University, Amsterdam, The Netherlands), it is important to mention here several generalizations regarding antibiotic susceptibility of the asaccharolytic BPBs: all are susceptible to penicillin, clindamycin, erythromycin, metronidazole, and tetracycline, are less susceptible to vancomycin, spiramycin, and chloramphenicol, and are resistant to gentamicin (4, 5, 62, 104, 113, 184; van Winkelhoff, personal communication). β-Lactamase has not been detected in any of the asaccharolytic BPB species (69, 104). The susceptibility of the three asaccharolytic BPBs to selected dyes and other agents has also been determined (104; Table 1).

Notten et al. (122) have recently found that one antibiotype of *B. gingivalis* predominates in the mouth of an individual and that different subjects harbor different antibiotypes of *B. gingivalis*.

OUTLOOK AND CONCLUSIONS

Recent developments in molecular biology and gene cloning and the use of specifically tailored microbial mutants will permit us to investigate the questions relevant to the role of BPBs and other microorganisms in mixed infections. Importantly, these new technologies will allow the determination of the role of virulence factors of asaccharolytic BPBs in these infective processes. Once the mutants are available, we will then need to test and compare them in animal models so that definitive proof of their role as specific virulence factors in disease initiation and tissue destruction can be obtained. In recent years, a considerable body of research has focused on the expression of potential virulence factors in vitro. *B. gingivalis*, for example, has an extensive proteolytic activity which includes both natural and artificial substrates. However, it is not known whether these activities are the result of a limited number of enzymes or if this bacterium has a larger set of enzymes, each with a specific range of substrates. Also, very little is known of the involvement of these proteolytic enzymes in situ. This can be said for most, if not all, of the factors affecting virulence of these bacteria.

Similarly, recently developed monoclonal antibodies (15, 47, 117, 158) will contribute to our knowledge of the localization of specific bacteria both in plaque samples and within tissues. These new approaches will be central to investigations into the activities of cell surface molecules (i.e., LPS, pili, outer membrane proteins, etc.) as colonization factors, proteases, and hemagglutinins on the surface of the asaccharolytic BPBs. Structures such as vesicles produced by all three asaccharolytic BPB species also need to be thoroughly investigated. Studies are needed to determine whether these structures are important in disease, to biochemically characterize the vesicles, to determine whether they are a mechanism by which *Bacteroides* species can exchange genetic material as in *Haemophilus* spp. (64) or whether they are a mechanism by which bacterial cells simply concentrate and secrete proteolytic enzymes. The results of these studies should help us to understand the role of these bacteria in mixed infections. Finally, we will need to gain more information specifically on the ecological distribution of *B. asaccharolyticus* and *B. endodontalis* and the role that they play in mixed infections.

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ADDENDUM IN PROOF

Love and co-workers (D. N. Love, J. L. Johnson, R. F. Jones, and A. Calverly, J. Bacteriol. **37**:307–309, 1987) recently described a new species of asaccharolytic black-pigmented *Bacteroides* isolated from cats, *B. salivosus*. This species produces catalase, does not agglutinate sheep erythrocytes, has a trypsinlike enzyme activity, and produces large quantities of phenylacetic acid. Vitamin K and hemin are both required for growth.

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